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## The effect of acetate on population heterogeneity in different cellular characteristics of *Escherichia coli* in aerobic batch cultures

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### Abstract

Acetate as the major by-product in industrial scale bioprocesses with *E. coli* is found to decrease process efficiency as well as to be toxic to cells, which has several effects like a significant induction of cellular stress responses. However, the underlying phenomena are poorly explored. Therefore we studied time-resolved population heterogeneity of the *E. coli* growth reporter strain MG1655/pGS20PrnBGFPAAV expressing destabilized green fluorescent protein during batch growth on acetate and glucose as sole carbon sources. Additionally, we applied five fluorescent stains targeting different cellular properties (viability as well as metabolic and respiratory activity). Quantitative analysis of flow cytometry data verified that bacterial populations in the bioreactor are more heterogeneous in growth as well as stronger metabolically challenged during growth on acetate as sole carbon source, compared to growth on glucose or acetate after diauxic shift. Interestingly, with acetate as sole carbon source significant subpopulations were found with some cells that seem to be more robust than the rest of the population. In conclusion, following batch

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cultures population heterogeneity was evident in all measured parameters. Our approach enabled a deeper study of heterogeneity during growth on the favoured substrate glucose as well as on the toxic by-product acetate. Using a combination of activity fluorescent dyes proved to be an accurate and fast alternative as well as a supplement to the use of a reporter strain. However the choice of combination of stains should be well considered depending on which population traits to aim for.

**Keywords:** Flow cytometry, reporter strain, growth on acetate, population heterogeneity, metabolic activity, viability

## Introduction

Nowadays it is known, that microbial cell populations in industrial scale, biotechnological production processes, though originating from pure, isogenic cultures, are heterogeneous. Due to non-ideal mixing, gradients of process parameters like substrate, dissolved oxygen and pH arise, creating different local microenvironments that are experienced by cells traveling throughout the reactor<sup>1-4</sup>. This induces dynamic cell responses on both genetic, metabolic and physiological level, and consequently causes development of population heterogeneity<sup>5-7</sup>. This extrinsic population heterogeneity may lead to reduced yields and productivity as well as elevated by-product formation<sup>2,3,8</sup>. Further intrinsic heterogeneity is added through stochastic converge and reaction of intracellular molecules<sup>9,10</sup>.

For instance, formation of acetate as the major by-product in industrial scale bioprocesses with *Escherichia coli* with glucose as carbon source, was found to decrease the biomass yield due to repeated production/re-assimilation when the cells move from zones with high glucose and low oxygen concentration to zones with low glucose and high oxygen saturation<sup>11,12</sup>. Hence, zones with different acetate concentration are formed, which is undesirable as acetate concentrations  $> 2 \text{ g.L}^{-1}$  are already toxic for cells<sup>13</sup>. Furthermore, acetate has several negative effects on growth and recombinant protein production as well as induces the cellular stress response, not the least because *E. coli* adjusts its expression pattern to growth on a secondary carbon source and vice versa<sup>14-17</sup>. Hence, potentially less productive or less robust subpopulations can occur that reduce overall

process efficiency. Even though these consequences of population heterogeneity and acetate formation are well-known, the underlying cellular phenomena are poorly understood<sup>4</sup>. One reason might be that studies of the role of acetate and diauxic shift have mostly been performed in shake flasks instead of bioreactors, or have focused on population level not taking into account single cell physiology<sup>18-20</sup>. This points to the need for investigating single cells physiology, especially the dynamics of cell viability and robustness of *E. coli* cells growing on acetate as sole carbon source as well as on acetate after diauxic shift in bioreactors<sup>12</sup>. Based on a deeper understanding of the differences between single cells in a population, strategies for process optimization, favouring subpopulations with beneficial characteristics may be developed.

Nevertheless, ordinary bioprocess monitoring often relies solely on population-based average measurements which do not distinguish co-existing cell behaviors, and are thus unable to reflect population heterogeneity among individual cells<sup>21,22</sup>. To discover and monitor cell-to-cell differences, cellular mechanisms and regulatory circuits at single cell level flow cytometry (FC) is nowadays the method of choice<sup>22,23</sup>. To visualize population heterogeneity in bioprocesses, fluorescent stains as well as reporter strains, expressing a fluorescence protein whose expression can be correlated to a gene product of interest<sup>4,22,24,25</sup>, are utilized. Furthermore, application of a combination of two or more fluorescent stains simultaneously unveils functional differences between bacterial cells, and as such population heterogeneity<sup>26,27</sup>. This enables the study of the physiological state of bacteria with a high resolution achieving distributions of the measured parameters, which has already been applied to study heterogeneity of microbial populations grown in batch cultures<sup>28,29</sup>. In this way this method is also advantageous compared to global omics-methods because of its simplicity, high-throughput and because some omics-methods are not yet available at single cell level<sup>4</sup>.

A big variety of fluorescence stains targeting different cellular properties are commercially available. The redox dye, 5-cyano-2,3-ditolyl tetrazolium chloride (CTC), is reduced by electron transport activity to a water insoluble, intracellular red fluorescent formazan and has routinely been used to monitor respiratory activity of cells<sup>30-32</sup>. The still rarely applied stain, redox sensor green (RSG) is a vitality stain that becomes green fluorescent when altered by bacterial reductases<sup>33</sup>. As many of these are part of the electron transport system, this stain can also be employed as an

indicator for respiratory ability of bacteria, which has also already been proven during studies of single cell physiology in bioreactors<sup>33,34</sup>. The membrane permeable, asymmetrical cyanine dye, SYBR<sup>®</sup> green, binds readily to nucleic acids in cells and has been applied in FC studies to, among others, quantify bacteria in crude environmental samples<sup>35,36</sup>. As a counterpart to activity measurements, the propidium iodide (PI) dye has commonly been used as membrane integrity indicator. It is a membrane impermeable nucleic acid stain and therefore employed to detect bacterial cells with disrupted membranes, as has already been demonstrated in several studies of bioprocesses<sup>4,37</sup>. Cells that are metabolically inactive lack the ability to exchange ions through the membrane, due to lack of membrane potential. The bis-(1,3-dibarbituric acid)-trimethine oxanol (DiBAC<sub>4</sub>(3)) dye has the ability to enter depolarized cells and, while retained inside, turns green<sup>38</sup>. Potential cell-to-cell heterogeneities in bioreactors may also be investigated by, as mentioned above, using the microbial population itself as reporter<sup>4,22,39</sup>. The green fluorescent protein (GFP) is a small bioluminescent protein that is widely used as promoter activity reporter in many bacteria and yeast cells as it does not interfere with cellular growth<sup>40,41</sup>. The high stability of the wild type GFP might pose a problem if transient gene expression is studied. Non-active, non-viable or dormant microbial cells would continue to fluoresce over many cell generations, even in absence of promoter gene expression<sup>42</sup>. It has been reported that the addition of an AAV-tag to the C-terminal of template GFP<sub>mut3.1</sub>, reduces its half-life from 24 h to 60 min, forming a destabilized GFP version<sup>43</sup>. An *E. coli* growth reporter with a destabilized GFP variant (AVV) fused to the ribosomal promoter *rrnB* P1P2<sup>44</sup> was used in this study. This promoter is known to be growth rate regulated and responsive to nutrient starvation<sup>45</sup>.

This study aimed at investigating the effect of acetate on physiological heterogeneity and viability of *E. coli*, which was examined in batch cultures under two different conditions; 1) while growing on acetate as sole carbon source, thus eliminating the effect of glucose; 2) during acetate consumption after diauxic shift. Additionally, single cell physiology was compared to growth on glucose. To assess population heterogeneity, a combination of five different fluorescent stains, CTC, RSG, SYBR<sup>®</sup> green, DiBAC<sub>4</sub>(3) and PI, with an *E. coli* growth reporter strain expressing a destabilized GFP was applied. Some stains targeted similar physiological characteristics of *E. coli*.

This redundancy enabled to draw conclusions about which stains are recommendable for investigation of population heterogeneity in bioprocesses.

The change of the state of individual cells in the microbial population throughout the batch experiment was analyzed by FC. The resulting multidimensional distribution data were quantitatively analyzed with a combination of cluster analysis and the relationship between coefficient of variance and mean fluorescence intensity, thereby revealing information about potential subpopulations as well as differences in shape and fluorescence intensity throughout different phases of the batch processes.

The coefficient of variance can be correlated to noise in gene expression, which describes how expression of two identical copies of a gene leads to deviations in different cells of an isogenic population (intrinsic noise), respectively is influenced by interaction of the cell with its environment (extrinsic noise)<sup>46,47</sup>. Noise in gene expression was found to be one major cause of population heterogeneity influencing bioprocess efficiency<sup>33,46,48</sup>. Upon occurrence, it leads to a broadening of distributions or multimodality<sup>49</sup>. Noise was found to be minimized in the expression of genes that are essential and contribute to cellular growth whereas it was elevated in genes that mediate the response to environmental changes e.g. the stress response, energy metabolism and carbon utilization<sup>50-52</sup>. Additionally, an inverse correlation between noise and gene expression could be established; highly expressed genes exhibit low levels of noise whereas the opposite was found for weakly expressed genes<sup>53</sup>. Therefore it was suspected that noise in gene expression is used as a regulatory strategy to increase respectively decrease the level of population heterogeneity depending on what is beneficial for the cell population<sup>47,48,53</sup>.

We hypothesize that acetate strongly affects the percentage of metabolically active cells and that population heterogeneity is used by the cells as a strategy to adjust culture robustness depending on the carbon source.

## Material and Methods

### Strains

A biosensor strain based on the expression of destabilized GFP under control of the *rrnBP1P2* promoter, MG1655/pGS20Pr*rrnBGFP*AAV, was used to study single cell growth dynamics<sup>44</sup>. The

recombinant strain MG1655/pGS20PrnB containing the same plasmid but lacking the *gfp* gene, was used as control strain of metabolic burden by expression of the fluorescent protein GFP and for staining with fluorescent stains to avoid interference with GFP fluorescence.

### **Preculture preparation**

Single colonies of MG1655/pGS20PrnBGFPAAV or the control strain from a Luria-Bertani (LB) plate were used to inoculate 100 mL LB broth in shaker flasks for growth overnight at 37 °C and 180 rpm. Serial dilutions in 10-fold steps were afterwards incubated in cultivation tubes at 37 °C for 6 to 8 h at 180 rpm in an orbital shaker. Cultures with optical density (OD<sub>600</sub>) 0.4–0.6 were used to inoculate bioreactors. The media were supplemented with 25 µg.mL<sup>-1</sup> chloramphenicol (dissolved in 96 % ethanol).

### **Batch cultivation**

Triplicate batch cultivations of MG1655/pGS20PrnBGFPAAV and MG1655/pGS20PrnB were performed in minimal medium<sup>54</sup> with 4 g.L<sup>-1</sup> glucose or 4 g.L<sup>-1</sup> acetate as sole carbon source. After autoclaving, 3 mL MgSO<sub>4</sub>, 1 mL trace metal solution and 25 µg.mL<sup>-1</sup> chloramphenicol were added by sterile filtration (pore size 0.2 µm). All cultivations were performed in 1 L bioreactors (Sartorius, Braun Biotech International GmbH, Germany) with a working volume of 1 L. Cultures were inoculated with OD<sub>600</sub> 0.005. Temperature, aeration and stirring were kept constant at 37 °C, 1 vvm and 1000 rpm, respectively, to avoid oxygen limitation. Sensors for pH and dissolved oxygen tension (Mettler Toledo, USA) were calibrated according to standard procedures provided by the manufacturer using a two-point calibration (pH 4 and 7, gassing with oxygen or nitrogen, respectively). Afterwards pH was controlled at 7 with 2 M NaOH and 2 M HCl. Continuous analysis of the off-gas composition was performed with a mass spectrometer (Prima Pro Process MS, Thermo Fisher Scientific, UK).

Samples for OD<sub>600</sub> measurements, high performance liquid chromatography (HPLC), dry cell weight (DW) and flow cytometry (FC) analysis were withdrawn periodically every 1.5 or 2 h. Samples for OD<sub>600</sub> and DW were analyzed directly, whereas filtrated samples for HPLC were kept at -20 °C until analysis. Samples for FC were kept on ice or at RT (see staining procedure).



### **OD<sub>600</sub>, DW and HPLC analysis**

Growth was followed with OD<sub>600</sub> measurements using a spectrophotometer (Shimadzu UV MINI 1240, Japan). Dry cell weight measurements were done with 5 mL of cultivation broth according to <sup>55</sup>. Concentrations of glucose, succinate, acetate, ethanol, glycerol, lactate, formate and pyruvate were determined by HPLC (Agilent 1100, Agilent Technologies, USA) with a 300 mm × 7.8 mm Aminex HPX-87H ion exchange column (Bio-Rad, Hercules, USA), refractive index detector (RID Agilent 1200, Agilent Technologies, USA) and UV detector (Agilent 1100, Agilent Technologies, USA) set to 210 nm. The mobile phase was 5 mM H<sub>2</sub>SO<sub>4</sub>, temperature 60 °C and flow rate 0.6 mL.min<sup>-1</sup>. The results from HPLC and dry cell weight measurements were used to calculate yield coefficients. All measurements were performed in triplicates.

### **Flow cytometry**

Bacterial cells were analyzed using a FACS Aria™ III (Becton Dickinson, USA) flow cytometer (FC). The system has two lasers, 488 nm and 561 nm, with minimum laser power of 10 mW. Two scattering channels, forward scatter (FSC) and side scatter (SSC), and two fluorescent detection channels (530/30 nm and 610/20 nm) were used in the analysis. For better visualization of the populations, the amplification voltages on the detectors were set based on negative and positive controls (stained, non-stained, viable and non-viable bacterial cells) to: FSC – 301 V; SSC – 316 V; 530/30 nm – 520 V and 610/20 nm – 582 V. To eliminate background noise, the detection thresholds were set in FSC and SSC. The FC specific software was used in combination with flow cytometer setup and tracking beads (Becton Dickinson, USA) for automated quality assurance and quality control of machine performance. All measurements were performed in triplicate and the obtained results were saved as fcs files.

### **Staining procedure**

Five different fluorescent dyes were used in this study: 5-cyano-2,3-ditolyltetrazolium chloride (CTC) (Sigma-Aldrich, Germany), SYBR<sup>®</sup> Green (SYBR<sup>®</sup>) (Invitrogen, USA), propidium iodide (PI) (Sigma-Aldrich, Germany), bis-(1,3-dibutylbarbituric acid) trimethine oxonol (DiBAC<sub>4</sub>(3)) (Molecular probes, USA) and redox sensor green (RSG) (Invitrogen, USA). For all stains 1 µL stain



was added to 100  $\mu\text{L}$  sample suspended in 0.9 % saline solution. The final stain concentrations were 1x for SYBR<sup>®</sup>, 0.05  $\text{mg}\cdot\text{mL}^{-1}$  for PI, 0.5 mM for CTC and 1  $\mu\text{M}$  for DiBAC<sub>4</sub>(3) and RSG.

To minimize the influence of sample handling prior to staining and during subsequent FC analysis, a validation procedure was performed (see supplementary material S1). Based on the results, adequate incubation times and temperatures were chosen. Samples to be stained with CTC were kept at room temperature (RT) until FC measurement and were incubated for 1 h at 37 °C during staining. Samples stained with DiBAC<sub>4</sub>(3), PI, RSG respectively SYBR<sup>®</sup> were incubated for 10 min respectively 20 min at RT. For PI, RSG and SYBR<sup>®</sup> samples were kept on ice until FC measurement. For DiBAC<sub>4</sub>(3) staining, samples collected during the first 10 h of batch process were stored at RT, thereafter on ice. All incubations were done in the dark. Based on the correlation between OD<sub>600</sub> and the number of events recorded in the FC, samples with OD<sub>600</sub> > 0.2 were diluted 1:10 before staining.

Additionally, staining variation in technical and biological replicates was evaluated in respective control experiments with the control strain MG1655/pGS20PrnB (see supplementary material S2).

### **Data analysis**

FC raw data processing and analysis was performed in MATLAB<sup>®</sup> R2017b (The MathWorks, Inc., Natick, USA). Fcs files, containing data for FSC, SSC, GFP and staining fluorescence, were loaded into MATLAB<sup>®</sup> using the readfcs function (by L. Balkay, University of Debrecen, Hungary, available on MATLAB<sup>®</sup> central file sharing) and saved into mat files. By applying the MATLAB<sup>®</sup> built-in hist function to the 1024 recording channels of the FC, cell count was saved for all channels and plotted as cell count against fluorescence intensity for each detector. The function FindGate (by M. McClean, Princeton University, USA, available on MATLAB<sup>®</sup> central file sharing) was used to gate for the main bacterial size population which compromised 95 % of the 10.000 events collected. Further, to account for heterogeneity in distributions of cellular properties the coefficient of variance (CV), defined as the ratio between standard deviation and mean of a distribution, was determined for distributions of GFP fluorescence and stains during growth on glucose, acetate and acetate after diauxic shift. The MATLAB<sup>®</sup> built-in mean and std function were used to calculate the

respective mean and standard deviation. Afterwards, figures for the relationship between heterogeneity and mean fluorescence were created.

To compare the shape of distributions for GFP and stains, hierarchical cluster analysis using the MATLAB<sup>®</sup> built-in linkage function was performed. The output matrix was used to create dendrogram plots for GFP and stains with the MATLAB<sup>®</sup> built-in dendrogram function. For a more distinct division of sub-clusters, this analysis was different than the comparison of noise and mean fluorescence, only performed with samples from exponential growth on glucose and acetate, respectively acetate after diauxic shift.

Where significant subpopulations of cells appeared in histogram plots, the subpopulation percentage was computed by dividing the histogram plot into areas of low, middle and high fluorescence. The areas were set in relation to the local minimum between the two subpopulations, determined with the MATLAB<sup>®</sup> built-in min function. To avoid biased data, the middle fluorescence range was excluded. The subpopulation percentage in the low and high range was calculated by dividing the cell amount in the respective subpopulation by the total cell amount collected in the sample.

## Results

To study the influence of acetate on the development of population heterogeneity, which is crucial in large-scale bioprocesses with *E. coli*, five different fluorescent stains in combination with a *E. coli* reporter strain for single cell growth were applied in laboratory scale batch cultures with glucose respectively acetate as sole carbon source respectively during acetate growth after diauxic shift. The five fluorescent stains targeted different cellular properties, respiratory ability (CTC and MTT), membrane integrity (DiBAC<sub>4</sub>(3) and PI) and DNA/RNA content (SYBR<sup>®</sup> green), related to the physiological cell status. The reporter strain was based on the expression of destabilized GFP (AAV) under control of the ribosomal *rrnBP1P2* promoter and therefore enabled the investigation of heterogeneities in the growth rate of single cells. To ensure that the expression of the fluorescent protein is not a burden to the cells the same reporter strain without the *gfp* gene was grown in batch culture on glucose. Furthermore, the control strain was also stained with the five stains to avoid interference of staining fluorescence with the GFP signal of the growth reporter strain.

Next to general physiological characterization, single cell fluorescence of the reporter strain and stains was followed during the different phases of the batch cultures using FC.

### Population level growth physiology

To account for population level physiology of the reporter and the control strain, yields of biomass, CO<sub>2</sub> and, for glucose cultivations, acetate on the substrate (glucose or acetate) were calculated based on data from biological triplicates (Table 1). All yields for glucose cultivations were in agreement with earlier studies<sup>54,56</sup> and no physiological differences between the reporter and the control strain could be found (Table 1). Carbon balance closed within  $1.05 \pm 0.00$  and  $1.06 \pm 0.04$ , for the control and the reporter strain, respectively. They also exhibited similar maximum specific growth rates during growth on glucose ( $\mu_{\max, \text{control}} = 0.72 \text{ h}^{-1} \pm 0.05$ ;  $\mu_{\max, \text{reporter}} = 0.70 \text{ h}^{-1} \pm 0.03$ ). Consequently, expression of GFP did not seem to be a burden to the cells. Both strains showed a typical growth behavior on glucose (reporter: Figure 1A; control: data not shown). After a phase of slow growth of around 6 h, an exponential growth phase with glucose consumption accompanied by the production of acetate, biomass and CO<sub>2</sub> followed (Table 1 and Figure 1A). With glucose depletion after around 10 h, the cells underwent diauxic shift, upon which the prior produced acetate was consumed. In this phase biomass was further accumulated accompanied by a rise in CO<sub>2</sub>, though with a lower rate compared to when glucose was the substrate ( $\mu_{\max, \text{acetate}} = 0.1 \text{ h}^{-1} \pm 0.02$  for both strains). Stationary phase was reached after around 14 h. No further metabolites were detected.

When acetate (Figure 1B) was sole carbon source both strains exhibited a longer phase of slow growth (around 17 h) compared to growth on glucose. Afterwards acetate was consumed accompanied by biomass and CO<sub>2</sub> accumulation until depletion after around 35 h. The maximum specific growth rate ( $\mu_{\max, \text{control}} = 0.15 \text{ h}^{-1} \pm 0.04$ ;  $\mu_{\max, \text{reporter}} = 0.14 \text{ h}^{-1} \pm 0.05$ ) as well as the biomass and CO<sub>2</sub> production were lower, resulting in around 50 % respectively 66 % lower biomass and CO<sub>2</sub> yield compared to growth on glucose (Table 1). However, values were comparable with growth on acetate after diauxic shift (Table 1). No other metabolites could be detected. In carbon balances around 61 % of the carbon was missing, though the biomass yield was consistent with earlier studies<sup>56,57</sup>. In HPLC chromatograms, an unidentified peak was seen, which may be

oxaloacetate (data not shown). Possibly, oxaloacetate was over-produced through the bypass of isocitrate dehydrogenase in acetate grown cultures, and was central to successful adaption and growth on acetate<sup>58</sup>.

### **Single cell level physiology**

Single cell level physiology was accessed using flow cytometry measurement of GFP fluorescence of the growth reporter strain and the five fluorescence stains. The resulting fluorescence distributions were analyzed calculating, supplementary to the mean fluorescence, the coefficient of variance (CV) as a measure of population heterogeneity which was suggested and applied as investigation of the mean to variation relationship in other publications<sup>33,46</sup>. Furthermore the CV can also provide information about the reproducibility of the performed cultivations and thereby the suitability of the applied stains for investigation of population heterogeneity in bioprocesses.

For exponential growth on both carbon sources as well as acetate after diauxic shift, additionally cluster analysis of the single cell distributions was performed. To our knowledge this is the first time that this method is applied for the analysis of population heterogeneity in bioprocesses. Cluster analysis is used to group single cell distributions that are more similar to each other in shape and fluorescence intensity in the same cluster compared to other distributions that exhibit different characteristics and are consequently clustered more distant respectively in another group. In this way, this exploratory data-mining method allows a facilitated and objective analysis of underlying connections between single cell distributions.

When there was an indication of the appearance of subpopulations, the relative fraction of cells in each subpopulation was quantified for samples taken following the batch.

### **Growth related fluorescence during growth on glucose and acetate**

Growth related heterogeneities in batch cultures with glucose or acetate as sole carbon source as well as during acetate growth after diauxic shift were followed by GFP expression of the growth reporter strain. Mean GFP expression (Figure 1A and B) was generally rather low throughout the cultivations, which also has been observed earlier<sup>44</sup>. However, a similar trend mirroring different growth phases for glucose and acetate grown cells could be observed. The fluorescent signal

increased during exponential growth phase and decreased again towards the end of the cultivation (Figure 1A and B). For cultivations with glucose as sole carbon source, mean fluorescence already decreased slightly during growth on acetate after diauxic shift (mean  $\pm$  standard deviation (std):  $194 \pm 10.87$ ) compared to exponential growth on glucose (mean  $\pm$  std:  $212.46 \pm 37.69$ ). However, growth on acetate after diauxic shift was showing slightly lower mean fluorescence values than for exponential growth on acetate as sole carbon source (mean  $\pm$  std:  $220 \pm 21.36$ ). When normalizing mean fluorescence values with the optical density of the culture the difference between cells grown on acetate as sole carbon source (normalized mean  $\pm$  std:  $116.10 \pm 19.58$ ) and on glucose as sole carbon (normalized mean  $\pm$  std:  $72.82 \pm 23.37$ ) increases as possibly acetate grown cells are smaller than cells growing on glucose.

The trend that the GFP fluorescence intensity is similar for growth on glucose and acetate after diauxic shift but different from growth on acetate as sole carbon source is also confirmed by cluster analysis (Figure 2A). Samples from batch cultivations with acetate as sole carbon source settle in a cluster distant from other samples. However, growth on glucose and acetate growth after diauxic shift still exhibit significant differences in fluorescence intensity and shape illustrated by different sub-clusters for the respective samples (Figure 2A). Surprisingly, no subpopulation division in slower and faster growing cells was found for any of the growth conditions, a phenomenon that has been suggested to occur in other studies<sup>59-61</sup>.

Population heterogeneity, described by the coefficient of variance of population distributions, is highest for cells grown on acetate as sole carbon source (Figure 3A). During growth on glucose respectively acetate after diauxic shift, population variation is around 40 % lower than during growth on acetate as sole carbon source. The only exception is one sample for growth on glucose from the beginning of the exponential growth phase that exhibits about the same level of variation as for growth on acetate as sole carbon source, which might be correlated to a widening of the distribution and thus more heterogeneity at the beginning of the exponential growth phase (Figure 3A). Afterwards, the level of variation is slightly decreasing during exponential growth on glucose whereas it remains about constant for growth on acetate as sole carbon source or after diauxic shift. This might be connected to a constant distribution shape (Supplementary material S3).

Apparently, cells growing on acetate as sole carbon source are more heterogeneous and possibly use noise in gene expression, which can be correlated with a change in distribution width, as a strategy to cope with the non-preferred carbon source acetate as sole carbon source as also proposed by <sup>51,62</sup>. In contrast, cells grown on acetate after diauxic shift seemed to be better prepared for growth on acetate and do not exhibit a rise in heterogeneity.

### **Physiological status evaluation by fluorescent staining**

To gain further insight into the physiological state of single cells during growth on acetate as sole carbon source or after diauxic shift in comparison to glucose and to assess the effect acetate has on the degree of population heterogeneity, a combination of five fluorescent stains were applied targeting the single cells' membrane integrity and chosen metabolic activities. Using redundant stains targeting similar characteristics, also the suitability of the respective stains for investigation of population heterogeneity in bioprocesses was investigated.

### **Membrane status assessment**

Cellular membrane integrity was assessed using PI and DiBAC<sub>4</sub>(3). For both stains a very low percentage of positive stained cells were detected (around 4-6 %), hence indicating that cells had predominantly intact membranes and maintained their membrane potential for all growth conditions applied in this study. However, the amount of PI positive cells showed a slight increase at the beginning of the exponential growth phase accompanied with a broadening of the peak for both, cells growing on acetate respectively glucose as sole carbon source, whereas this increase was not observed when cells grew on acetate after diauxic shift (Supplementary material S3). This change in membrane integrity is also confirmed by a 21 % respectively 26 % higher level of heterogeneity (CV) in samples for exponential growth on glucose respectively acetate as sole carbon source compared to growth on acetate after diauxic shift. For cells stained with DiBAC<sub>4</sub>(3) no increase at the beginning of the exponential growth phase can be detected, so that glucose grown cells show a similar level of heterogeneity to cells growing on acetate after diauxic shift. Additionally, although generally distributions for both stains show the same trend, distributions for cells stained with PI were less reproducible than for cells stained with DiBAC<sub>4</sub>(3) as manifested in a slightly higher standard deviation between triplicate measurements, which however is consistent with the staining

variation found in control experiments so that an experimental artifact can be excluded (data not shown, and supplementary material S2).

### **Metabolic activity assessment**

For assessment of metabolic heterogeneity the DNA/RNA stain SYBR<sup>®</sup> green and two vitality stains, CTC and RSG, were applied.

### **Respiratory ability – CTC**

Fluorescence distribution profiles for cells stained with CTC exhibited distinct differences for cells growing on the two different carbon sources. However, the distance between the samples taken during growth on different carbon sources is smallest compared to all other stains applied (Figure 2). Samples from pure acetate culture clustered closely together and separate from the rest of the samples for growth on glucose respectively growth on acetate after diauxic shift (Figure 2B). Consequently, respiratory ability during growth on acetate after diauxic shift seems to be similar to growth on glucose. However, the Euclidean distance is bigger to cells sampled from the beginning of the exponential growth phase than to later samples. A similar trend is seen for growth on acetate as sole carbon source (Figure 2B). For samples taken towards the end of the exponential growth phase the Euclidean distance is higher compared to respiratory ability at the beginning of the exponential growth phase. This might be connected to an increased level of heterogeneity in respiratory ability towards the end of exponential growth on both carbon sources respectively during growth on acetate after diauxic shift. This trend can also be confirmed by the coefficient of variance that increases for samples taken towards the end of the cultivation for both carbon sources as well as acetate growth after diauxic shift (Figure 3B).

The coefficient of variance was generally higher for growth in pure acetate cultures compared to growth on glucose (Figure 3B). At the same time the mean shows the opposite trend being highest for the first samples during growth on glucose as sole carbon source especially in the beginning of the exponential growth phase and lowest for exponential growth on acetate (Figure 3B). This is in accordance with the hypothesis that the level of population heterogeneity is higher in weaker expressed functions than for stronger expressed functions<sup>46,51,53</sup>. Interestingly, when cells grew on acetate after diauxic shift the variation level was lower and the mean fluorescence was higher, and therefore resembled glucose grown cells.



However these differences between the two cultures might also be connected to the finding that for cells from pure acetate cultures, the CTC distributions displayed a small subpopulation exhibiting a higher respiratory activity than the main population (Figure 4A). The subpopulation distribution remained constant during the batch, with the low fluorescent main population, comprehending around 90 % of the cells and the small fraction of cells with a higher fluorescence (around 10 %) (Figure 4A). This subpopulation was neither present during exponential growth on glucose nor during acetate growth after diauxic shift. Furthermore, for the main population, a lower fluorescence signal was observed for cells grown on acetate compared to cells grown on glucose (Figure 3B and supplementary material S3).

### **Respiratory ability - RSG**

When staining with RSG as for staining with CTC, differences in respiratory ability between cells growing on the two carbon sources were observed. However no clear distinction of cells growing on acetate or glucose as well as acetate after diauxic shift was possible when applying cluster analysis, as the Euclidean distance between some samples taken for growth on the same carbon source had a considerable size (Figure 2C). Nevertheless, sub-clusters can be identified. The first sub-cluster includes samples for exponential growth on glucose and a sample for advanced exponential growth on acetate as sole carbon source. These samples probably exhibit a similar distribution shape and in comparison to other samples mid-fluorescence intensity (Figure 3C and supplementary material S3). Similarly, the third sub-cluster contains samples for growth on glucose and acetate as well as one sample for growth on acetate after diauxic shift, which show a similar level of variation and mean respiratory ability (Figure 3C).

The remaining samples for early exponential growth on acetate settle in the second sub-cluster (Figure 2C). As for CTC, a small subpopulation was seen for growth on acetate as sole carbon source, but only at the beginning of the growth curve (Figure 4B). The relative fraction of the low fluorescent subpopulation increased during exponential growth from around 80 % to almost 100 % with acetate depletion, when the high fluorescent subpopulation disappeared. Due to the appearance of the high fluorescent subpopulation these samples exhibited a significantly higher mean fluorescence than other samples for growth on acetate as sole carbon source as well as growth on glucose or acetate after diauxic shift (Figure 3C). Generally mean respiratory ability for cultivations

on both carbon sources shifted up at the start of the batch and decreased again over time. However, respiratory ability for growth on acetate as sole carbon source remained always higher than for growth on glucose or especially acetate after diauxic shift (Figure 3C).

The coefficient of variance was about constant and highest for growth on acetate after diauxic shift. For growth on acetate as sole carbon source slightly lower variation levels were found, which were higher in the beginning of the culture co-occurring with the small subpopulation (Figure 3C and Figure 4B) as well as towards the end of the cultivation. A similar trend was seen for cells growing on glucose as sole carbon source during which generally the lowest levels of variation were found. Apparently, cells growing on acetate as sole carbon source as well as acetate after diauxic shift are more heterogeneous in respiratory ability and possibly use population heterogeneity as a strategy to cope with acetate as also proposed elsewhere<sup>51,62</sup>.

Considering the reproducibility of the measurements, standard deviations for cells stained with RSG were around 10 - 15 %, especially during growth on acetate as sole carbon source, indicating less reproducible staining results between replicates as also confirmed in control experiments prior to the study (Figure 3C and supplementary material S2).

#### **DNA/RNA content - SYBR®**

Also for SYBR® fluorescence, which can be used to access the DNA/RNA content on single cell level, distributions for growth on the two carbon sources were not clearly distinguishable in cluster analysis (Figure 2D). Hence, there seems to be increased heterogeneity in the fluorescence level and bigger changes following exponential growth on glucose and acetate as sole carbon source as well as acetate after diauxic shift. However, a general trend can be extracted with samples from growth on acetate as sole carbon source clustering in a middle range, surrounded by samples from growth on acetate after diauxic shift (Figure 2D). Samples for growth on glucose as sole carbon source built the outer layer of the cluster. Consequently, the DNA/RNA content for growth on acetate as sole carbon source and acetate after diauxic shift seem to be similar, however not significantly different to growth on glucose. During exponential growth on acetate as sole carbon source the DNA/RNA content and distribution shape seem to fluctuate, so that no clear order of samples in the cluster could be identified (Figure 2D). For cells grown on glucose, the DNA/RNA content was high at the beginning, but decreased close to diauxic shift and further during growth on acetate after diauxic

shift reaching its minimum towards the end of the cultivation prior to transition to stationary phase when also the coefficient of variance levels rises, while the shape probably remained similar (Figure 2D and 3D). Compared to growth on glucose, the mean DNA/RNA content was generally lower for growth on acetate as sole carbon source as well as during acetate consumption after diauxic shift (Figure 3D). For SYBR no distinct subpopulation division was visible; rather the whole population was moving (Supplementary material S3).

Considering the coefficient of variance (Figure 3D), it is clear that the cells grown on acetate as sole carbon source as well as after diauxic shift yield more heterogeneity and are more similar in DNA/RNA content in comparison to growth on glucose. For growth on acetate after diauxic shift, the highest variation levels are found, potentially because cells already prepare for stationary phase. For some samples the standard deviations for calculations of the coefficient of variance for SYBR® were around 15 % indicating low reproducibility between replicates thus a slightly higher staining heterogeneity as found in preliminary experiments (Supplementary material S2).

## Discussion

In the present study five different stains targeting metabolic activity and viability in combination with a GFP-growth reporter strain were successfully applied for single cell analysis and mapping of population distributions over time in *E. coli* batch cultivations on acetate and glucose as sole carbon sources as well as during acetate growth after diauxic shift. All stains were able to monitor population heterogeneity, however with different accuracy. As a consequence, below some recommendations for the use of fluorescent stains will be given.

To our knowledge, for the first time the effect of acetate was examined in more detail at single cell level. In traditional studies, heterogeneity of microbial populations is often not investigated at the single cell level. Instead, one usually relies on data averaged across thousands or millions of cells in a sample <sup>4,5</sup>. To enable the development of robust high-yielding bioprocesses it is however important to also improve the understanding of microbial population heterogeneity and how population dynamics are influenced by changes in environmental conditions, especially when potentially toxic by-products are formed <sup>22</sup>.

### **Influence of acetate on population heterogeneity in different growth phases**

Growth of *E. coli* on acetate is inhibited even at very low concentrations of 0.5 g.L<sup>-1</sup> <sup>63</sup>. This inhibition becomes very significant in a defined medium when acetate is sole carbon source <sup>64</sup>. It is known that bacterial populations become more asynchronous under stress and contain cells at all stages of the division cycle <sup>65</sup> among others due to an increase in noise in gene expression leading to a higher level of heterogeneity <sup>48</sup>. Hence, our hypothesis was that the enhanced stress during growth on acetate would lead to increased heterogeneity levels of the population, compared to growth on glucose.

Our data support this, as in general more heterogeneous population distributions were observed for acetate grown cells especially when acetate was sole carbon source. However, the differences in population heterogeneity between the two carbon sources were smaller than expected, which is in agreement with earlier findings <sup>66,67</sup>. Comparing acetate growth after diauxic shift to growth on acetate as sole carbon source the population distributions exhibited similar traits. However, growth related fluorescence during growth on acetate after diauxic shift was more similar to single cell growth on glucose and no subpopulation development was detected as for growth on acetate as sole carbon source. Interestingly, a small portion of cells that are more robust and tolerant to acetate stress seem to exist, since this subpopulation with higher respiratory activity than the main population were found. But these cells might not be able to withstand acetate stress for the whole batch process, as for one of the applied respiratory ability stain the subpopulation disappeared in mid-exponential phase. Also this subpopulation of cells does not appear during growth on acetate after diauxic shift.

Furthermore, *E. coli* cells seem to use population heterogeneity as a strategy to prepare for growth, as seen by a higher level of variation at the beginning of the exponential growth phase on both carbon sources. In addition population heterogeneity apparently helps to cope with the non-preferred carbon source acetate as manifested in a higher constant variation level and also proposed by others <sup>51,62</sup>.

In comparison cells grown on acetate after diauxic shift seemed to be better prepared for growth on acetate probably due to rearrangements taking place once glucose is depleted, as seen by a change in DNA/RNA content during diauxic shift. Consequently, no significant lag-phase during diauxic

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shift is observed for any of the measured single cell characteristics. However compared to cells grown on acetate as sole carbon source, cells grown on acetate after diauxic shift seem to exhibit slightly lower vitality as well as a lower growth rate on population and single cell level. Apparently, during the relatively fast diauxic shift cells are primed to adjust their metabolism in a focused way whereas during growth on acetate as sole carbon source the cells are less focused and rather try to be prepared for as many situations as possible, which is supported by a higher level of heterogeneity. Nevertheless, with this 'broader response' also some cells arise that can cope better with the presence of acetate than the remaining population which increases the overall fitness of the population similar to what was suggested for heterogeneity of the bacterial growth rate <sup>25</sup>. Furthermore, this is in agreement with findings that when a lag-phase that involves adjustment to new environmental conditions appears, a fraction of the cells might be better adjusted and able of producing an immediate response <sup>60</sup>.

Moreover, it is interesting to note that regardless of the carbon source, hardly any cells with compromised or depolarized membrane (data from PI and DiBAC<sub>4</sub>(3) staining), and therefore regarded as non-viable or dormant, were detected. Consequently, considering the long phase of slow growth in the acetate cultivations, cells seem to be stressed and have to adjust to the harsh growth conditions, but the acetate does not significantly influence the viability of the cells.

### **Connection observed between metabolic activity and growth physiology of the reporter strain**

In glucose cultures, the cells appeared to respire more actively and synthesize nucleic acids prior to exponential growth phase. Afterwards, activities generally decrease in exponential phase and after diauxic shift reaching a constant level with the onset of stationary phase. The same trend could also be followed with the growth reporter strain. GFP fluorescence increased with the beginning of the exponential phase on glucose, indicating active synthesis of ribosomal RNA, before declining during acetate growth and reaching a plateau with the beginning of the stationary phase. Different from earlier studies of the diauxic shift of other *E. coli* strains in shake flasks, no subpopulation division in single cell growth was found upon diauxic shift from growth on glucose to growth on acetate <sup>59</sup>. Reasons for that might be that MG1655 can adjust more efficiently to new environmental

conditions, possibly due to its high flexibility in metabolism <sup>68</sup>, and the controlled environment of the bioreactor.

Discrepancies were found between the results obtained with CTC and RSG. Although both strains are used to detect respiratory activity, the CTC distributions exhibited more heterogeneity than the RSG distributions. The distinct difference between acetate and glucose grown cells seen for cells stained with CTC, i.e. more heterogeneous distribution seen for cells from acetate cultivations, in addition to the existence of a small subpopulation with a higher respiratory level, was only observed during the beginning of the exponential growth phase when staining with RSG. However, in general both stains showed a slightly lower respiratory activity when growing on acetate than during growth on glucose. This might be connected to the lower growth rate on acetate, which implies a slower metabolism and thereby lower respiratory activity. This is also supported by the results for SYBR<sup>®</sup>, demonstrating that cells exhibit a higher rate of DNA/RNA production when growing with a higher growth rate on glucose than when growing on acetate. The growth reporter strain revealed slightly higher normalized mean GFP fluorescence levels during exponential growth on acetate compared to glucose. This fact is contradictory to the observation of the higher fluorescent signal for SYBR<sup>®</sup> from cells grown on glucose than from cells grown on acetate and to the generally accepted belief that fast growing cells have a higher number of ribosomal operons resulting in a higher synthesis rate of rRNA than cells growing at a slower growth rate<sup>34</sup>. One explanation might be that glucose exhibits a repression effect on the ribosomal activity. Potentially the strain's machinery is working harder to cope with the harsh conditions in the presence of acetate, as reflected in higher average ribosomal activity and the RSG and SYBR<sup>®</sup> data. Further investigations are required to fully understand the mechanisms involved in these phenomena.

### **Conclusions and recommendations with regards to the usefulness of the applied stains**

In the present study five different fluorescence stains targeting respiratory ability, DNA/RNA content and membrane integrity of which some were redundant, have been applied leading to partially different results and accuracy (for a summary and comparison see Table 2).

Respiratory ability was assessed with two stains, CTC and RSG. CTC has frequently been used to define the amount of “active” bacteria in samples of diverse origin <sup>69,70</sup>. So far no direct correlation

between the amount of cells that are actively respiring, growing and dividing and the amount of cells that reduced enough dye to be detected could be verified. However a good correlation between colony forming units (CFU) and CTC positive cells regardless of the growth phase was found <sup>71</sup>. But the use of CTC is controversial since a possible toxic effect to the cells due to lowered counts of positive cells compared to other studies cannot be avoided. Moreover an unified protocol for the application of CTC is missing which complicates the comparison of results reported in different publications <sup>72</sup>.

RSG is altered by active reductases in the cells <sup>33</sup>, some of which are part of the electron transport system <sup>34</sup>. Therefore it enables the assessment of bacterial respiratory and metabolic activity, also in bioprocesses, which was already proven earlier <sup>33</sup>. Furthermore, for the application of the stain a standardized protocol exists. Compared to CTC, RSG showed lower staining reproducibility and sensitivity in our study especially when expecting higher respiring subpopulations, why the choice of which one to utilize should be taken with care depending on the application.

DNA/RNA content was evaluated by staining with SYBR<sup>®</sup>. This stain exhibited a bright signal and high sensitivity capturing fluctuations during the different phases of the batch cultures on both carbon sources. But, especially during growth on acetate as sole carbon source staining reproducibility was lower than for the other stains. However, due to consistency of changes in DNA/RNA content found with physiology on population and single cell level, SYBR<sup>®</sup> can still be recommended.

The two stains targeted to access membrane integrity, PI and DiBAC<sub>4</sub>(3), demonstrated very low, not significant, fractions of positively stained cells. However, it was noticed that the percentage of PI positive cells increased at the onset of exponential growth for both growth on glucose and on acetate. These observations appear to corroborate the existence of false positives when bacteria are actively growing as previously observed in environmental bacterial samples <sup>73</sup>, and considering that PI false positives have also been detected in stressed cultures of *S. cerevisiae* <sup>74</sup>. In conclusion, PI should be used with care when assessing viability of cells under dynamic growth conditions. Consequently, DiBAC<sub>4</sub>(3) that shows highly reproducible results, is sensitive however does not seem to be prone to false positives, and may therefore be more suitable as a vitality indicator in batch cultures (for a summary of properties see Table 2).



### **Potential of the combined use of reporter strains together with stains**

The physiological state of microorganisms in bioprocesses can be affected by a number of environmental factors, which consequently may influence process performance. A thorough understanding of the state of individual cells is thus needed to achieve high process efficiency. Traditional cultivation data reflect the general metabolic ability of production strains but do not illustrate single cell differences. Using rapid fluorescent staining procedures and reporter strains combined with FC provided fast information on cellular physiological status changes and population distributions. It has to be mentioned, that the use of reporter strains might not be possible in an industrial bioprocess as the production host cannot be genetically altered, however just using several fluorescence stains in parallel would increase the information about the single cells' physiological state in a bioprocess significantly. Furthermore, although this approach was comprehensive, just choosing one of the stains in combination with the growth reporter strain and analyze the distributions quantitatively would already reveal significant information about population heterogeneity and provide an important supplement to the traditional methods used in industry to monitor batch cultures. The stains form an alternative to study populations until global omics-methods become available on single cell level.

### **Conclusion**

Especially when gradients of process parameters occur caused by non-ideal mixing in large scale bioprocesses with *E. coli*, acetate is a major, potentially toxic by-product. The present study aimed at investigating the effect of acetate on single cell physiology of *E. coli* in batch processes applying five fluorescence stains targeting respiratory and metabolic activity as well as viability in combination with a growth reporter strain. Batch growth on acetate as sole carbon was compared to growth on glucose as well as acetate growth after diauxic shift using cluster analysis and examining the relationship between the coefficient of variance and mean fluorescence for the reporter strain and the different stains. Differences between growth on glucose and acetate exist, however they are smaller and less significant than expected, revealing some cells that are more respiratory active than the main population during growth on acetate as sole carbon source. Furthermore population heterogeneity was found to be applied as a strategy to cope with acetate. Consequently, the

combination of stains and reporter strains is a sensible approach to increase the knowledge of single cell physiology during bioprocesses with *E. coli*.

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## Literature cited

1. Lara AR, Leal L, Flores N, Gosset G, Bolivar F, Ramirez OT. Transcriptional and metabolic response of recombinant *Escherichia coli* to spatial dissolved oxygen tension gradients simulated in a scale-down system. *Biotechnol Bioeng*. 2006;93(2):372-385.
2. George S, Larsson G, Olssen K, Enfors SO. Comparison of the Baker's yeast process performance in laboratory and production scale. *Bioprocess Engineering*. 1998;18:135-142.
3. Larsson G, Törnkvist M, Stahl Wernersson E, Tragardh C, Noorman H, Enfors SO. Substrate gradients in bioreactors: origin and consequences. *Bioprocess Engineering*. 1996;14:281-289.
4. Heins AL, Weuster-Botz D. Population heterogeneity in microbial bioprocesses: origin, analysis, mechanisms, and future perspectives. *Bioprocess Biosyst Eng*. 2018.
5. Avery SV. Microbial cell individuality and the underlying sources of heterogeneity. *Nat Rev Microbiol*. 2006;4(8):577-587.
6. Delvigne F, Zune Q, Lara AR, Al-Soud W, Sorensen SJ. Metabolic variability in bioprocessing: implications of microbial phenotypic heterogeneity. *Trends Biotechnol*. 2014;32(12):608-616.
7. Simsek E, Kim M. The emergence of metabolic heterogeneity and diverse growth responses in isogenic bacterial cells. *ISME J*. 2018;12(5):1199-1209.
8. Bylund F, Collet E, Enfors SO, Larsson G. Substrate gradient formation in the large-scale bioreactors lowers cell yield and increases by-product formation. *Bioprocess Engineering*. 1998;18:171-180.
9. Binder D, Drepper T, Jaeger KE, et al. Homogenizing bacterial cell factories: Analysis and engineering of phenotypic heterogeneity. *Metab Eng*. 2017;42:145-156.
10. Kiviet DJ, Nghe P, Walker N, Boulineau S, Sunderlikova V, Tans SJ. Stochasticity of metabolism and growth at the single-cell level. *Nature*. 2014;514(7522):376-379.
11. Enfors SO, Jahic M, Rozkov A, et al. Physiological responses to mixing in large scale bioreactors. *Journal of biotechnology*. 2001;85:175-185.

12. Luli GW, Strohl WR. Comparison of Growth, Acetate Production, and Acetate Inhibition of Escherichia coli Strains in Batch and Fed-Batch Fermentations. *Applied and Environmental Microbiology*. 1990;56(4):1004-1011.
13. Gschaedler A, Robas N, Boudrant J, Branlant C. Effects of Pulse Addition of Carbon Sources On Continuous Cultivation of Escherichia coli Containing a Recombinant E. coligapAGene. *Biotechnology and Bioengineering*. 1999;63(6).
14. Shiloach J, Fass R. Growing E. coli to high cell density—A historical perspective on method development. *Biotechnology Advances*. 2005;23(5):345-357.
15. Eiteman MA, Altman E. Overcoming acetate in Escherichia coli recombinant protein fermentations. *Trends Biotechnol*. 2006;24(11):530-536.
16. Fernandez-Coll L, Cashel M. Contributions of SpoT Hydrolase, SpoT Synthetase, and RelA Synthetase to Carbon Source Diauxic Growth Transitions in Escherichia coli. *Front Microbiol*. 2018;9:1802.
17. Wolfe AJ. The acetate switch. *Microbiol Mol Biol Rev*. 2005;69(1):12-50.
18. Enjalbert B, Coccagn-Bousquet M, Portais JC, Letisse F. Acetate Exposure Determines the Diauxic Behavior of Escherichia coli during the Glucose-Acetate Transition. *J Bacteriol*. 2015;197(19):3173-3181.
19. Enjalbert B, Letisse F, Portais JC. Physiological and Molecular Timing of the Glucose to Acetate Transition in Escherichia coli. *Metabolites*. 2013;3(3):820-837.
20. Liu M, Durfee T, Cabrera JE, Zhao K, Jin DJ, Blattner FR. Global transcriptional programs reveal a carbon source foraging strategy by Escherichia coli. *J Biol Chem*. 2005;280(16):15921-15927.
21. Dhar N, McKinney JD. Microbial phenotypic heterogeneity and antibiotic tolerance. *Current Opinion in Microbiology*. 2007;10(1):30-38.
22. Fernandes RL, Nierychlo M, Lundin L, et al. Experimental methods and modeling techniques for description of cell population heterogeneity. *Biotechnol Adv*. 2011;29(6):575-599.
23. Fritsch FS, Dusny C, Frick O, Schmid A. Single-cell analysis in biotechnology, systems biology, and biocatalysis. *Annu Rev Chem Biomol Eng*. 2012;3:129-155.
24. Delvigne F, Pecheux H, Tarayre C. Fluorescent Reporter Libraries as Useful Tools for Optimizing Microbial Cell Factories: A Review of the Current Methods and Applications. *Front Bioeng Biotechnol*. 2015;3:147.
25. Takhaviev V, Heinemann M. Metabolic heterogeneity in clonal microbial populations. *Curr Opin Microbiol*. 2018;45:30-38.
26. Nebe-von-Caron G, Stephens PJ, Hewitt CJ, Powell JR, Badley RA. Analysis of bacterial function by multi-colour fluorescence flow cytometry and single cell sorting. *Journal of microbiological methods*. 2000(42):97-114.
27. Hewitt CJ, Nebe-von-Caron G, Nienow A, McFarlane C. The use of multi-parameter flow cytometry to compare the physiological response of Escherichia coli W3110 to glucose limitation during batch, fed-batch and continuous culture cultivations. *Journal of biotechnology*. 1999(75):251-264.
28. Quiros C, Herrero M, Garcia LA, Diaz M. Application of flow cytometry to segregated kinetic modeling based on the physiological states of microorganisms. *Appl Environ Microbiol*. 2007;73(12):3993-4000.
29. Rezaeinejad S, Ivanov V. Heterogeneity of Escherichia coli population by respiratory activity and membrane potential of cells during growth and long-term starvation. *Microbiol Res*. 2011;166(2):129-135.

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30. Prorot A, Eskicioglu C, Droste R, Dagot C, Leprat P. Assessment of physiological state of microorganisms in activated sludge with flow cytometry: application for monitoring sludge production minimization. *J Ind Microbiol Biotechnol*. 2008;35(11):1261-1268.
  31. Rodriguez GG, Phipps D, Ishiguro K, Ridgway HF. Use of a fluorescent redox probe for direct visualization of actively respiring bacteria. *Applied and Environmental Microbiology*. 1992;58(6).
  32. Belkova NL, Tazaki K, Zakharova JR, Parfenova VV. Activity of bacteria in water of hot springs from Southern and Central Kamchatskaya geothermal provinces, Kamchatka Peninsula, Russia. *Microbiol Res*. 2007;162(2):99-107.
  33. Baert J, Delepierre A, Telek S, et al. Microbial population heterogeneity versus bioreactor heterogeneity: Evaluation of Redox Sensor Green as an exogenous metabolic biosensor. *Engineering in Life Sciences*. 2016;16(7):643-651.
  34. Kalyuzhnaya MG, Lidstrom ME, Chistoserdova L. Real-time detection of actively metabolizing microbes by redox sensing as applied to methylotroph populations in Lake Washington. *ISME J*. 2008;2(7):696-706.
  35. Zipper H, Brunner H, Bernhagen J, Vitzthum F. Investigations on DNA intercalation and surface binding by SYBR Green I, its structure determination and methodological implications. *Nucleic Acids Res*. 2004;32(12):e103.
  36. Noble RT, Fuhrman JA. Use of SYBR Green I for rapid epifluorescence counts of marine viruses and bacteria. *Aquatic microbial ecology*. 1998;14:113-118.
  37. Sträuber H, Müller S. Viability states of bacteria--specific mechanisms of selected probes. *Cytometry A*. 2010;77(7):623-634.
  38. Laflamme C, Lavigne S, Ho J, Duchaine C. Assessment of bacterial endospore viability with fluorescent dyes. *Journal of Applied Microbiology*. 2004;96(4):684-692.
  39. Delvigne F, Brognaux A, Francis F, et al. Green fluorescent protein (GFP) leakage from microbial biosensors provides useful information for the evaluation of the scale-down effect. *Biotechnol J*. 2011;6(8):968-978.
  40. Sorensen SJ, Burmolle M, Hansen LH. Making bio-sense of toxicity: new developments in whole-cell biosensors. *Curr Opin Biotechnol*. 2006;17(1):11-16.
  41. Andersen JB, Sternberg C, Kongsbak Poulsen L, Petersen Bjorn S, Givskov M, Molin S. New unstable variants of green fluorescent protein for studies of transient gene expression in bacteria. *Applied and Environmental Microbiology*. 1998;64(6):2240-2246.
  42. Leveau JH, Lindow SE. Predictive and interpretive simulation of green fluorescent protein expression in reporter bacteria. *J Bacteriol*. 2001;183(23):6752-6762.
  43. Miller WG, Leveau JH, Lindow SE. Improved gfp and inaZ Broad-Host-Range Promoter-Probe Vectors. *MPMI*. 2000;13(11):1243-1250.
  44. Han S, Delvigne F, Brognaux A, Charbon GE, Sorensen SJ. Design of growth-dependent biosensors based on destabilized GFP for the detection of physiological behavior of Escherichia coli in heterogeneous bioreactors. *Biotechnol Prog*. 2013;29(2):553-563.
  45. Jin DJ, Cagliero C, Zhou YN. Growth rate regulation in Escherichia coli. *FEMS Microbiol Rev*. 2012;36(2):269-287.
  46. Taniguchi Y, Choi PJ, Li G-W, et al. Quantifying *E. coli* proteome and transcriptome with single-molecule sensitivity in single cells. *Science*. 2011;329:533-538.
  47. Liu J, Francois JM, Capp JP. Use of noise in gene expression as an experimental parameter to test phenotypic effects. *Yeast*. 2016;33(6):209-216.

48. Delvigne F, Baert J, Sassi H, Fickers P, Grünberger A, Dusny C. Taking control over microbial populations: Current approaches for exploiting biological noise in bioprocesses. *Biotechnol J*. 2017.
49. Lemoine A, Delvigne F, Bockisch A, Neubauer P, Junne S. Tools for the determination of population heterogeneity caused by inhomogeneous cultivation conditions. *J Biotechnol*. 2017;251:84-93.
50. Swain PS, Elowitz MB, Siggia ED. Intrinsic and extrinsic contributions to stochasticity in gene expression. *PNAS*. 2002;99(20):12795-12800.
51. Elowitz MB, Levine AJ, Siggia ED, Swain PS. Stochastic gene expression in a single cell. *Science*. 2002;297:1183-1186.
52. Silander OK, Nikolic N, Zaslaver A, et al. A genome-wide analysis of promoter-mediated phenotypic noise in *Escherichia coli*. *PLoS Genet*. 2012;8(1):e1002443.
53. Baert J, Kinet R, Brognaux A, et al. Phenotypic variability in bioprocessing conditions can be tracked on the basis of on-line flow cytometry and fits to a scaling law. *Biotechnol J*. 2015;10(8):1316-1325.
54. Xu B, Jahic M, Blomsten G, Enfors SO. Glucose overflow metabolism and mixed-acid fermentation in aerobic large-scale fed-batch processes with *Escherichia coli*. *Appl Microbiol Biotechnol*. 1999(51):564-571.
55. Olssen L, Schulze U, Nielsen J. On-line bioprocess monitoring ^ an academic discipline or an industrial tool? *trends in analytical chemistry*. 1998;17(2).
56. Steinsiek S, Frixel S, Stagge S, Sumo, Bettenbrock K. Characterization of *E. coli* MG1655 and *frdA* and *sdhC* mutants at various aerobiosis levels. *J Biotechnol*. 2011;154(1):35-45.
57. Andersen KB, von Meyenburg K. Are growth rates of *Escherichia coli* in batch cultures limited by respiration? *Journal of bacteriology*. 1980;144(1):114-123.
58. Cozzone AJ, El-Mansi M. Control of isocitrate dehydrogenase catalytic activity by protein phosphorylation in *Escherichia coli*. *J Mol Microbiol Biotechnol*. 2005;9(3-4):132-146.
59. Kotte O, Volkmer B, Radzikowski JL, Heinemann M. Phenotypic bistability in *Escherichia coli*'s central carbon metabolism. *Mol Syst Biol*. 2014;10:736.
60. Boulineau S, Tostevin F, Kiviet DJ, ten Wolde PR, Nghe P, Tans SJ. Single-cell dynamics reveals sustained growth during diauxic shifts. *PLoS One*. 2013;8(4):e61686.
61. Labhsetwar P, Cole JA, Roberts E, Price ND, Luthy-Schulten ZA. Heterogeneity in protein expression induces metabolic variability in a modeled *Escherichia coli* population. *PNAS*. 2013;110(34):14006-14011.
62. Martins BM, Locke JC. Microbial individuality: how single-cell heterogeneity enables population level strategies. *Curr Opin Microbiol*. 2015;24:104-112.
63. Nakano K, Rischke M, Sato S, Märkl H. Influence of acetic acid on the growth of *Escherichia coli* K12 during high-cell-density cultivation in a dialysis reactor. *Appl Microbiol Biotechnol*. 1997;48:597-601.
64. Koh BT, Nakashimada U, Pfeiffer M, Yap MGS. Comparison of acetate inhibition on growth of host and recombinant *E. coli* K12 strains. *Biotechnology Letters*. 1992;14(12):1115-1118.
65. Gilbert P, Collier PJ, Brown MRW. Influence of Growth Rate on Susceptibility to Antimicrobial Agents: Biofilms, Cell Cycle, Dormancy, and Stringent Response. *Antimicrob Agents Chemother*. 1990;34(10):1865-1868.
66. Nikolic N, Barner T, Ackermann M. Analysis of fluorescent reporters indicates heterogeneity in glucose uptake and utilization in clonal bacterial populations. *BMC Microbiol*. 2013;13(258).
67. Nikolic N, Schreiber F, Dal Co A, et al. Cell-to-cell variation and specialization in sugar metabolism in clonal bacterial populations. *PLoS Genet*. 2017;13(12):e1007122.

68. Campbell K, Herrera-Dominguez L, Correia-Melo C, Zelezniak A, Ralser M. Biochemical principles enabling metabolic cooperativity and phenotypic heterogeneity at the single cell level. *Current Opinion in Systems Biology*. 2018;8:97-108.
69. Bartosch S, Mansch R, Knötzsch K, Bock E. CTC staining and counting of actively respiring bacteria in natural stone using confocal laser scanning microscopy. *Journal of Microbiological Methods*. 2003;52(1):75-84.
70. Servais P, Agogue H, Courties C, Joux F, Lebaron P. Are the actively respiring cells (CTC+) those responsible for bacterial production in aquatic environments? *FEMS Microbiol Ecol*. 2001;35:171-179.
71. Créach V, Baudoux A-C, Bertru G, Rouzic BL. Direct estimate of active bacteria: CTC use and limitations. *Journal of Microbiological Methods*. 2003;52(1):19-28.
72. Nielsen JL, Kragelund C, Nielsen PH. Combination of Fluorescence In Situ Hybridization with Staining Techniques for Cell Viability and Accumulation of PHA and polyP in Microorganisms in Complex Microbial Systems. In: Cummings SP, ed. *Bioremediation: Methods and Protocols*. Totowa, NJ: Humana Press; 2010:103-116.
73. Shi L, Günther S, Hübschmann T, Wick LY, Harms H, Müller S. Limits of propidium iodide as a cell viability indicator for environmental bacteria. *Cytometry A*. 2007;71(8):592-598.
74. Davey HM, Hexley P. Red but not dead? Membranes of stressed *Saccharomyces cerevisiae* are permeable to propidium iodide. *Environ Microbiol*. 2011;13(1):163-171.

**Table 1 - Yields of batch cultivations of MG1655/pGS20PrnBGFPAAV and MG1655/pGS20PrnB grown on glucose and acetate as sole carbon source as well as acetate after diauxic shift.**

<b>Yield</b>	<b>Control<sub>glucose</sub></b>	<b>Control<sub>acetate</sub></b>	<b>Biosensor<sub>glucose</sub></b>	<b>Biosensor<sub>acetate</sub></b>
Y <sub>SX</sub> [cmole/cmole] <sub>Total</sub>	0.65 ± 0.03	0.27 ± 0.03	0.64 ± 0.07	0.28 ± 0.02



$Y_{SX}$ [cmole/cmole] Glucose	$0.55 \pm 0.03$	n.a.	$0.56 \pm 0.09$	n.a.
$Y_{SX}$ [cmole/cmole] Acetate	$0.10 \pm 0.01$	n.a.	$0.08 \pm 0.01$	n.a.
$Y_{SC}$ [cmole/cmole]	$0.31 \pm 0.04$	$0.11 \pm 0.02$	$0.34 \pm 0.02$	$0.10 \pm 0.03$
$Y_{SC}$ [cmole/cmole] Glucose	$0.25 \pm 0.05$	n.a.	$0.28 \pm 0.02$	n.a.
$Y_{SC}$ [cmole/cmole] Acetate	$0.06 \pm 0.01$	n.a.	$0.06 \pm 0.01$	n.a.
$Y_{SAc}$ [cmole/cmole]	$0.08 \pm 0.02$	n.a.	$0.08 \pm 0.04$	n.a.

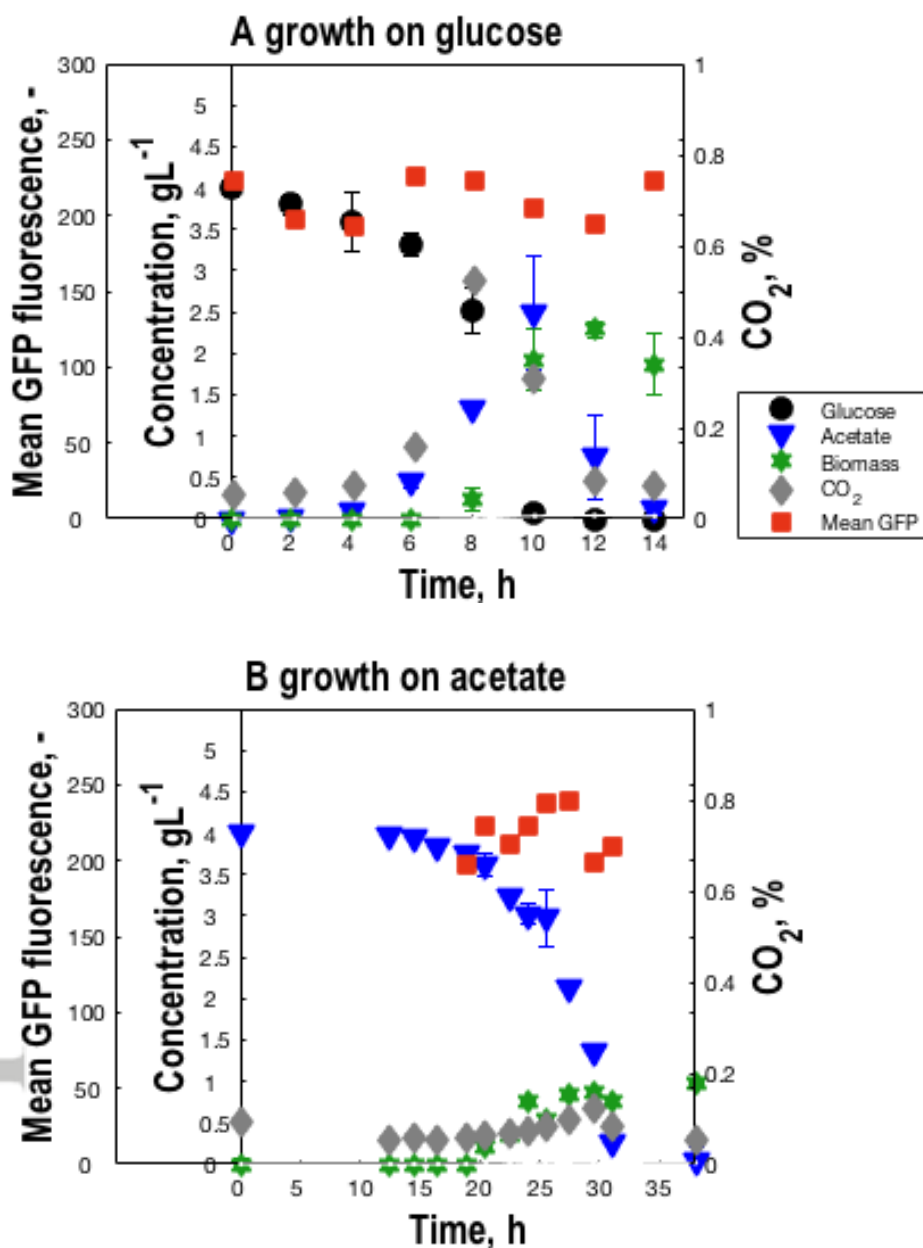
$Y_{SX}$ , growth yield on glucose respectively acetate;  $Y_{SC}$ , yield of  $CO_2$  on glucose respectively acetate;  $Y_{SAc}$ , yield of acetate on glucose; all values are given as mean with error bars originating from biological triplicates

**Table 2 - Comparison of different fluorescent stains used in this study.**

	<u>Stained property</u>	<u>Membrane integrity</u>		<u>Metabolic/ respiratory ability</u>		
	Applied fluorescent stain	PI	DiBAC <sub>4</sub> (3)	CTC	RSG	SYBR <sup>®</sup>
Characteristics	Sensitivity	+-	+	+	+-	+
	Reproducibility	+-	+	+	-	+-
	Defined protocol	+	+	-	+	+
	Fluorescence signal	+	+	+	+	+
	Interference (carbon source etc.)	+-	-	+-	n.k.	n.k.
	False positives	+	-	n.k.	n.k.	n.k.

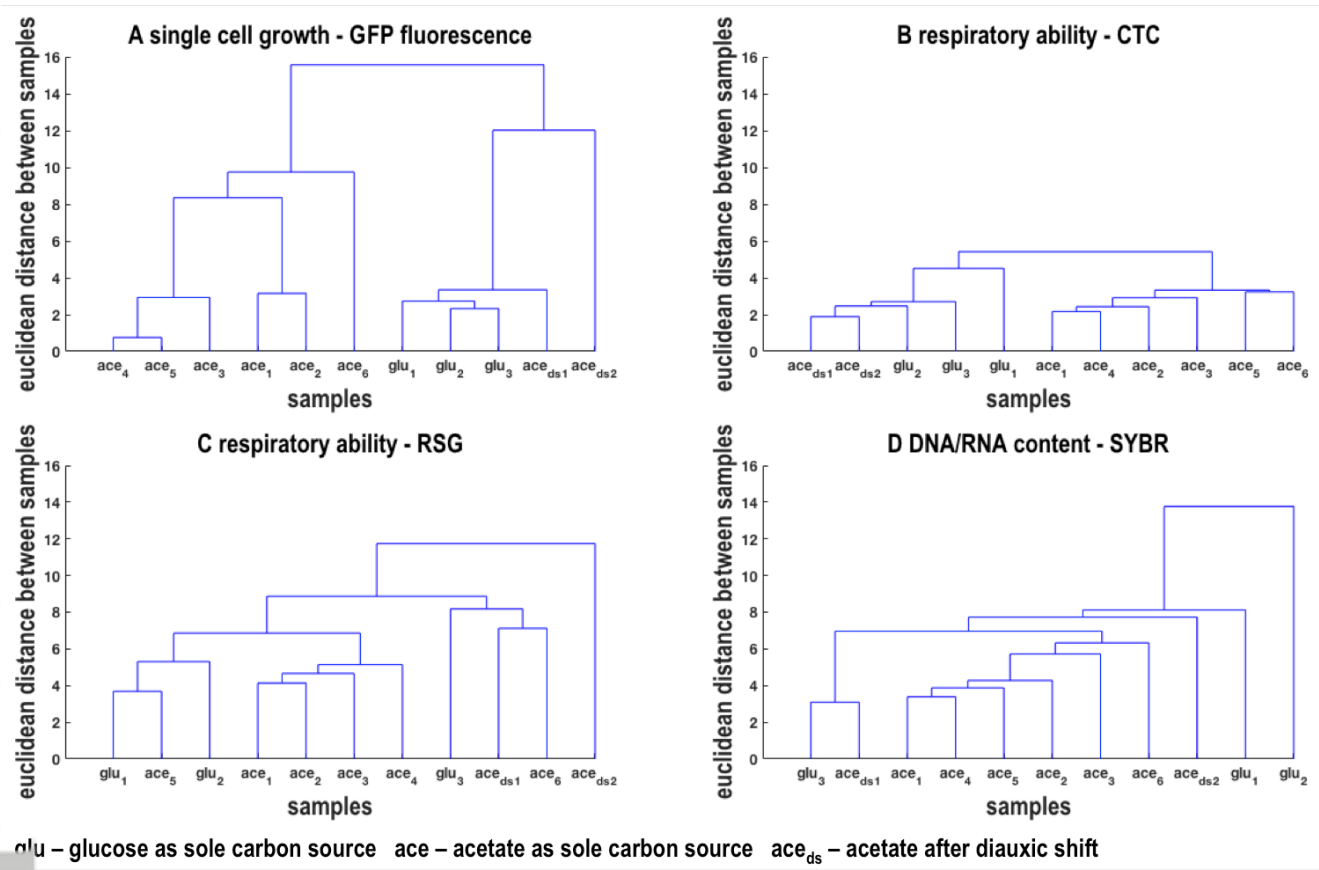
CTC, 5-cyano-2,3-ditolyl tetrazolium chloride; DiBAC<sub>4</sub>(3), bis-(1,3-dibarbituric acid)-trimethine oxanol; PI, propidium iodide; RSG, Redox Sensor Green; Rating is done by direct comparison of the applied stains



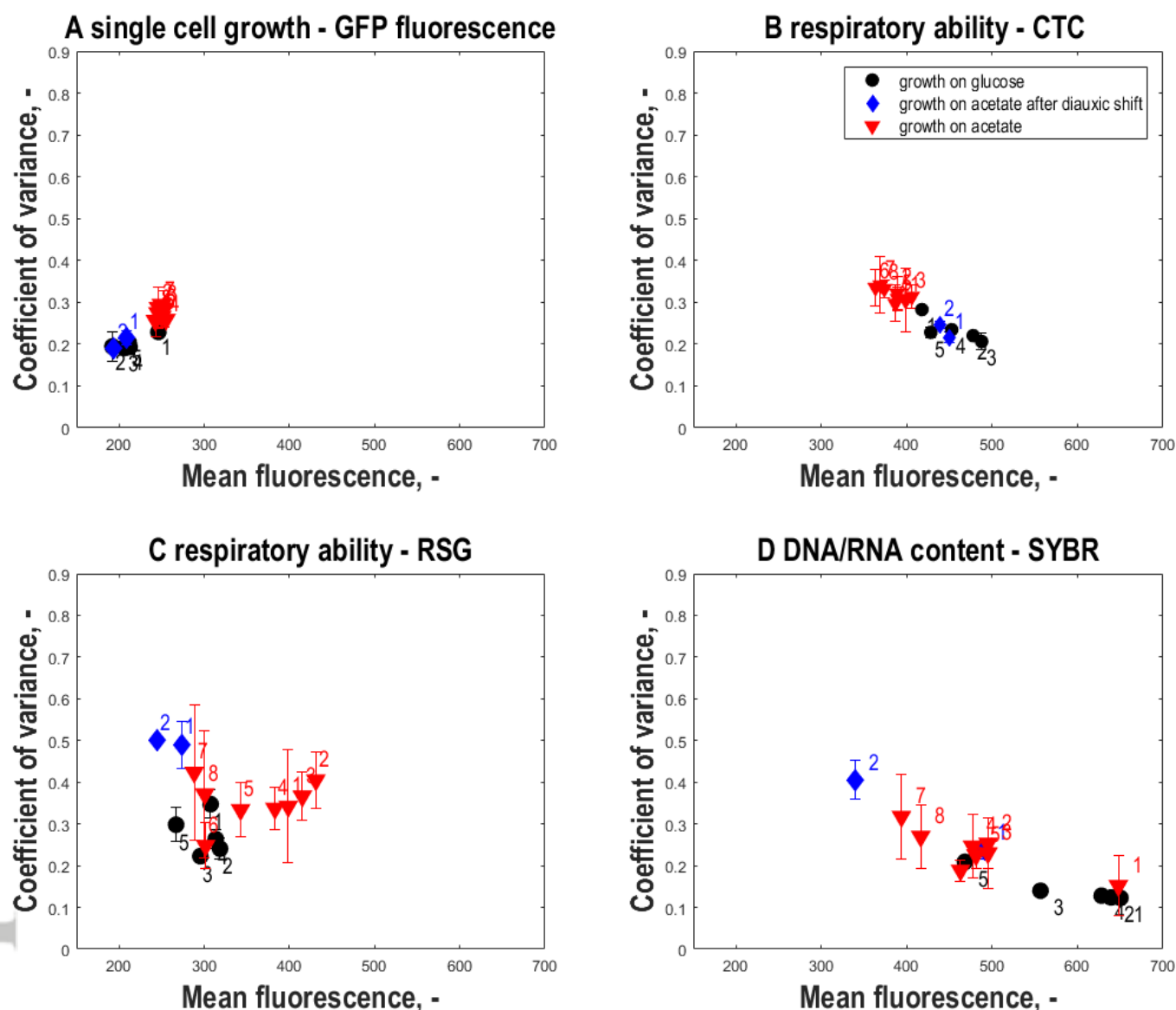


**Figure 1 - Physiology and mean GFP fluorescence for MG1655/pGS20PrnBGFPAAV in batch cultivations on glucose (A) or acetate as sole carbon source (B) showing glucose (g.L<sup>-1</sup>, circle), acetate (g.L<sup>-1</sup>, triangle), biomass (g.L<sup>-1</sup>, star), CO<sub>2</sub> (% , diamond) and mean GFP**

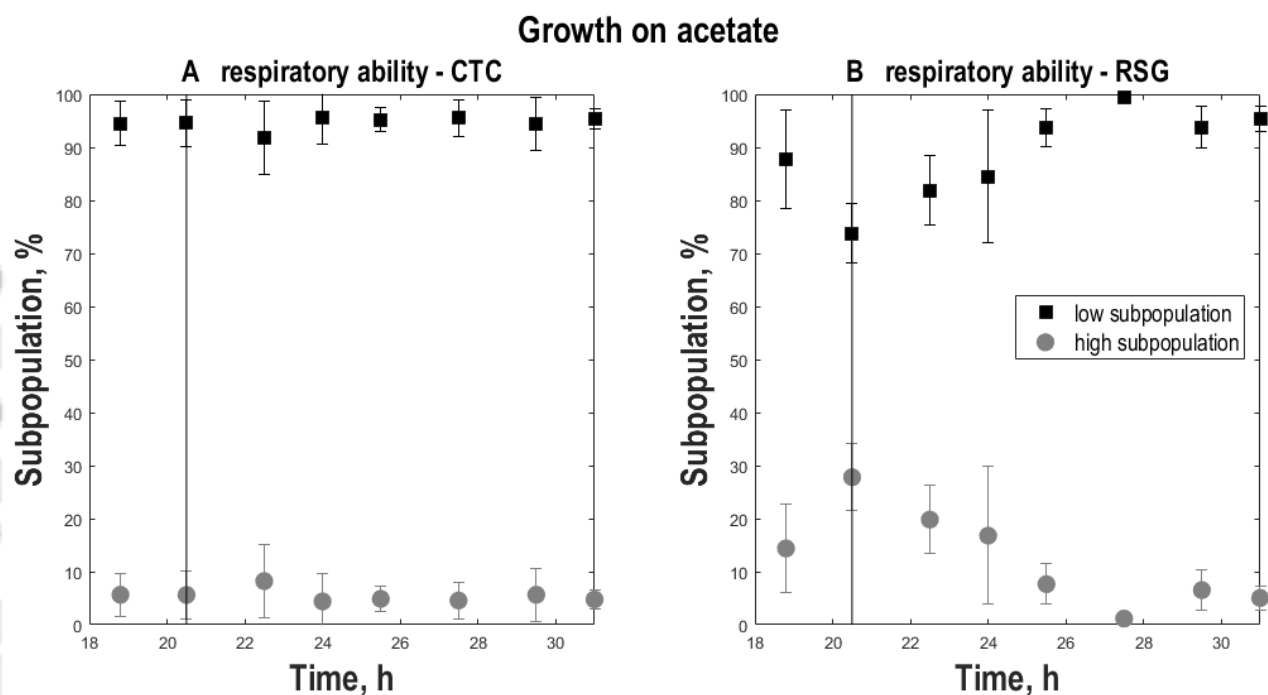
fluorescence (arbitrary units, square). Data represent averaged values with error bars originating from biological triplicates.



**Figure 2 – Euclidean distance between samples for growth related GFP fluorescence (A) of MG1655/pGS20PrnnBGFPAAV as well as MG1655/pGS20PrnnB stained with CTC (B), RSG (C) and SYBR (D) in batch cultivations during exponential growth on glucose or acetate as sole carbon source and during acetate growth after diauxic shift. Data represent averaged values originating from biological triplicates.**



**Figure 3 - Coefficient of variance vs. mean fluorescence for growth related GFP fluorescence (A) of MG1655/pGS20PrnBGFPAAV as well as MG1655/pGS20PrnB stained with CTC (B), RSG (C) and SYBR (D) in batch cultivations during growth on glucose (circle) or acetate (triangle) as sole carbon source and during acetate growth after diauxic shift (diamond). Data represent averaged values with error bars originating from biological triplicates.**



**Figure 4 - Subpopulation distribution for MG1655/pGS20PrnB cells stained with CTC (A) and RSG (B) in batch cultivations on acetate as sole carbon source showing high (circle) and low fluorescent populations (square). The black line indicates the beginning of the exponential growth phase on acetate. Data represent averaged values with error bars originating from biological triplicates.**